

# Correlation Between Genotype and Phenotype in Hereditary Hemochromatosis: Analysis of 61 Cases

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**ABSTRACT:** This report assesses the degree of iron overload in a cohort of patients in relationship to the presence or absence of the recently described 845 G→A (C282Y) and 187 C→G (H63D) mutations in the HFE (HLA-H) gene. Sixty-one patients with hereditary hemochromatosis diagnosed either with liver biopsy or on clinical grounds were included in this analysis. Forty-one patients were homozygous for C282Y, the genotype considered to be characteristic of hereditary hemochromatosis. At the time of this analysis, 37 of these 41 patients had achieved a state of iron depletion and mobilizable iron was calculated; 19 had less than 4 grams. Twenty-Five of these 41 patients had liver biopsies; 4 of these patients had a hepatic iron index less than 1.9. Of the 4 patients with a normal hepatic iron index, 3 had a quantitative hepatic iron of greater than 50 μmol/g dry weight, and one had an inadequate biopsy sample. These findings support our suspicion that individuals may have hereditary hemochromatosis and homozygous C282Y despite relatively low body iron stores.

Five patients were compound heterozygotes for C282Y and H63D. Four of these patients underwent liver biopsy; two had a hepatic iron index greater than 1.9. A third patient had a hepatic iron index of 1.3 but a quantitative hepatic iron of 90.6 μmol/g dry weight. All patients were phlebotomized to a state of iron depletion and only one of these patients had a mobilizable iron greater than 4 grams. Three patients were homozygous for H63D; these patients had either a hepatic iron index >1.9 or greater than 4 grams of mobilizable iron. Patients with homozygous H63D and significant iron overload are not well described. Seven patients were heterozygous for either C282Y or H63D; 4 had significant iron overload but three did not. Five patients had no HFE mutations; one of these patients unequivocally has iron overload with a hepatic iron index of 4.4.

We conclude that: (1) Identification of HFE mutations will be clinically useful in identifying patients with hereditary hemochromatosis, (2) Patient genotyping will help confirm a diagnosis of hereditary hemochromatosis in some patients with relatively low body iron stores, (3) Significant iron loading can occur in the absence of homozygous C282Y, adding to the evidence that genes other than HFE may be involved in iron loading, and (4) Homozygous H63D can be associated with significant iron overload.

Keywords: liver, iron, biopsy, transferrin saturation, HFE, HLA-H

## INTRODUCTION

Hereditary hemochromatosis is a disorder of iron metabolism associated with enhanced

gastrointestinal absorption of iron and eventual excessive tissue storage and dysfunction of affected target organs (1,2). The prevalence of hereditary hemochromatosis is 3-8 per 1,000

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(3,4), and it has been demonstrated that it is cost-effective to screen asymptomatic adults for this disorder (5,6). The diagnosis of hereditary hemochromatosis can be suspected based on serum iron studies (7,8,9,10), however, is usually confirmed with a liver biopsy and determination of quantitative hepatic iron (11,12,13). Whereas there is a poor correlation between the serum ferritin value and the quantitative hepatic iron, mobilizable iron stores can be used as a measure of body iron stores (14), and can in retrospect confirm a diagnosis of hereditary hemochromatosis. Because many patients do not meet established diagnostic cut-points yet may be at risk for iron accumulation and tissue damage, we offer phlebotomy treatments to all those with known or suspected hereditary hemochromatosis even if they do not meet strict criteria. It is anticipated that genetic testing will enhance the ability to diagnose hereditary hemochromatosis and may obviate the need for liver biopsy in many patients.

Recently, a candidate gene for hereditary hemochromatosis (designated HFE) was identified. Mutations within this gene may account for 80-90% of hereditary hemochromatosis cases (15,16,17). The purpose of this study was to determine the relationship between genotype (the presence of the HFE mutations), and phenotype (hepatic iron index and mobilizable iron) in a cohort of patients with hereditary hemochromatosis diagnosed by liver biopsy or other clinical parameters.

## PATIENTS AND METHODS

Our treatment center currently cares for 140 patients with hereditary hemochromatosis. This population includes patients with biopsy proven hereditary hemochromatosis and patients with a high likelihood of hereditary hemochromatosis based on clinical and laboratory parameters. The biopsy proven cases meet standard histologic and biochemical criteria. The clinically suspected cases include patients with elevated transferrin saturation and serum ferritin values who have no

known underlying disorder associated with iron loading, and are presumed to have hereditary hemochromatosis. This series focuses on 61 patients who are currently being treated in our center and have consented to have HFE genotyping.

Of these 61 patients, 37 have had a liver biopsy. The liver biopsies were performed using a 17-gauge Jamshidi needle, a standard transthoracic approach employing a Menghini aspiration technique to obtain the liver tissue. The specimens were sent for routine histological study and stained with hematoxylin and eosin, Massons trichrome, reticulin and Prussian blue. The stainable iron was characterized with a semi-quantitative grading system from grade 0-IV, and portion of the liver biopsy sample was used to measure the total iron in liver biopsy specimens spectrophotometrically. The quantitative hepatic iron was converted to a hepatic iron index (ratio of hepatic iron concentration in  $\mu\text{mol/g}$  dry weight to age). A hepatic iron index  $>1.9$  or a quantitative hepatic iron  $>50 \mu\text{mol/g}$  was the basis for making the diagnosis of hereditary hemochromatosis.

Fifty-one of the 61 patients have undergone therapeutic phlebotomy to achieve a state of iron depletion (serum ferritin  $<25 \text{ ng/ml}$ ), enabling use to determine their mobilizable iron stores. Our nursing staff have maintained records of their phlebotomy treatments in a data base, and the mobilizable iron was determined based on the blood volume removed prior to achieving a state of iron depletion using the following formula: grams of iron =  $\text{Hct}/3 \times \text{weight of blood} \times 0.0035$ .

All 61 patients have had HFE genotyping performed. After obtaining informed consent, blood was sent to the Centers for Disease Control and Prevention (lab of Dr. Chin-Yih Ou) for genotyping. Samples were shipped by overnight mail and processed within 24 hours upon their arrival to yield DNA and stored at  $-20\text{C}$ . A serial laboratory number was assigned to each participant's DNA and relevant information (i.e., Laboratory ID and genotypes) was stored in a Microsoft Excel database. The genetic testing

was performed using a well-characterized and standardized protocol developed and fully tested in the Molecular Biology Branch, Division of Environmental Health Science Laboratory, NCEH. A new method, known as TaqMan, was used to facilitate the hemochromatosis genotyping process. This state-of-the-art technology uses the 5' to 3' exonuclease activity of DNA Taq polymerase and two fluorescence-tagged DNA oligonucleotide probes to differentiate the wild type sequence and the mutant sequence during the DNA amplification process. The genotyping and amplification processes were carried out simultaneously in the Applied Biosystem's 7700 genotyper. The fluorescent output of probes was calculated by a specific, Excel-based software to define the three genotypes (homozygous normal, heterozygous, and homozygous mutation) of each locus. The

result were then transferred to the inventory database with only a few manual manipulations. The validity of this genotyping process has been extensively tested by comparing it with two other methods, i.e., direct DNA sequencing and single-stranded conformational polymorphism analysis. All three tests gave concordant results.

## RESULTS

A total of 61 patients with biopsy proven or clinically diagnosed hereditary hemochromatosis had HFE genotyping performed. Table 1 shows the distribution of genotypes in these patients and the mean hepatic iron index and mean mobilizable iron when these values were available. Table 2 shows the detailed analysis of these patients.

**Table 1.** Distribution of genotypes in 61 hereditary hemochromatosis patients

Genotype	C282Y Homozygote (845A/845A)	Compound Heterozygote (845A/187G)	H63D Homozygote (187G/187G)	C282Y Heterozygote (845A/+)	H63D Heterozygote (187C/+)	Normal +/+
<b>Total patients</b>	41/61 (67%)	5/61 (8.2%)	3/61 (4.9%)	4/61 (6.5%)	3/61 (4.9%)	5/61 (8.2%)
<b>Mean HII Range</b>	3.91 (n=25) 0.88-11.4	2.1 (n=4) 0.5-3.9	3.95 (n=2) 3.5, 4.4	0.87 (n=2) 0.84, 0.9	1.9 (n=2) 1.5, 2.3	2.5 (n=2) 0.58, 4.4
<b>Mean mobFe Range</b>	5.15 gm (n=37) 1.40-17.28	3.41 gm (n=5) 1.54-5.93	2.95 gm (n=2) 1.81, 4.08	2.51 gm (n=3) 1.23-4.19	6.83 gm (n=2) 2.31, 11.4	1.13 gm (n=2) 0.49, 1.77

HII = hepatic iron index

mobFe = mobilizable iron

+ = designates the normal 845G, 187C

patients within each column are not necessarily identical

**Table 2.** Laboratory features of patients with a clinical diagnosis of hereditary hemochromatosis who are homozygous H63D, heterozygous for C282Y or H63D, compound heterozygotes, or lack HFE mutations. Patient #3 was diagnosed and treated prior to arrival in our center.

	Quantitative Iron ( $\mu\text{mol/g}$ )	Hepatic Iron Index	Serum Ferritin	Transferrin Saturation	Mutation C282Y	Mutation H63D	Mobilized Iron (g)	Age
1			835	38	-/-	-/-		67
2			1792	97	-/-	-/-		56
3					-/-	-/-	1.77	44
4	22.7	0.58	1400	80	-/-	-/-	0.494	40
5	346.2	4.4	2246	44	-/-	-/-		80
6			1978	81	-/-	+/-	11.36	65
7	118.3	2.3	583	59	-/-	+/-		53
8	70.6	1.5	506	98	-/-	+/-	2.307	48
9			320	42	+/-	-/-	2.124	62
10	58.8	0.9	663	64	+/-	-/-	4.193	65
11			193	22	+/-	-/-	1.226	28
12	59.3	0.84	633	41	+/-	-/-		71
13	28	0.5	1327	58	+/-	+/-	3.716	55
14	100	2.7	795	40	+/-	+/-	2.978	38
15	180.7	3.9	1045	87	+/-	+/-	5.932	46
16	90.6	1.3	349	56	+/-	+/-	1.537	71
17					+/-	+/-	2.904	54
18	129	4.4	248	49	-/-	+/+	1.815	30
19	207.5	3.5	1000	87	-/-	+/+		61
20			1214	50	-/-	+/+	4.076	67

**Table 2 (Cont'd).** Laboratory features of patients with a clinical diagnosis of hereditary hemochromatosis who are homozygous C282Y. Patient #57 was diagnosed and treated prior to arrival in our center.

	Quantitative Iron ( $\mu\text{mol/g}$ )	Hepatic Iron Index	Serum Ferritin	Transferrin Saturation	Mutation C282Y	Mutation H63D	Mobilized Iron (g)	Age
21			183	87	+/+	-/-		30
22	184.4	4.09	1000	93	+/+	-/-	8.99	47
23	111.1	3.2	231	79	+/+	-/-	3.124	36
24			326	91	+/+	-/-	3.225	26
25	132.6	2.7	883	99	+/+	-/-	5.635	50
26			289	81	+/+	-/-	4.95	46
27			1965	97	+/+	-/-	10.184	58
28	109	2.02	478	70	+/+	-/-	3.195	55
29	203.2	5.9	1327	88	+/+	-/-	4.175	35
30	505.4	11.4	1694	95	+/+	-/-	17.283	47
31	59.9	2.2	525	85	+/+	-/-	0.726	28
32	9.7	0.2	640	68	+/+	-/-	2.737	40
33			372	63	+/+	-/-	2.214	55

	Quantitative Iron ( $\mu\text{mol/g}$ )	Hepatic Iron Index	Serum Ferritin	Transferrin Saturation	Mutation C282Y	Mutation H63D	Mobilized Iron (g)	Age
34			666	96	+/+	-/-	9.925	54
35	80	2.6	508	52	+/+	-/-	3.37	31
36	65.8	1.09	530	97	+/+	-/-	2.57	62
37	231.9	3.8	762	73	+/+	-/-	7.44	63
38			191	74	+/+	-/-	1.404	31
39	246.7	7.7	421	83	+/+	-/-	7.131	34
40	165.6	3.76	634	68	+/+	-/-	6.563	45
41			1030	99	+/+	-/-	3.439	81
42	52.1	0.88	774	97	+/+	-/-	3.207	60
43			296	67	+/+	-/-	3.413	57
44			2500	65	+/+	-/-	4.141	56
45			800		+/+	-/-		51
46			548	77	+/+	-/-	4.278	41
47			274	70	+/+	-/-	1.862	26
48	270.7	6.4	1934	100	+/+	-/-	10.228	44
49	102.1	2.3	439	87	+/+	-/-	2.927	46
50	76.8	1.6	1350	74	+/+	-/-	3.737	49
51			768	48	+/+	-/-	3.696	35
52	192.5	3	1007	100	+/+	-/-	6.168	65
53			1961	67	+/+	-/-	2.407	63
54	185.6	3.1	753	96	+/+	-/-	5.754	61
55	379.8	7.6	1280		+/+	-/-	13.072	51
56		2.3	1120	47	+/+	-/-	6.618	50
57					+/+	-/-		75
58	176.6	4.1	565	65	+/+	-/-	4.891	44
59	226.83	8.03	558	51	+/+	-/-		29
60	319	4.2	396	66	+/+	-/-	3.516	78
61	116.9	3.7	380	98	+/+	-/-	2.672	33

Forty-one patients were homozygous for C282Y, the genotype considered to be characteristic of hereditary hemochromatosis. At the time of this analysis, 37 of these 41 patients had achieved a state of iron depletion and mobilizable iron values were calculated; 19 had less than 4 grams of mobilizable iron. These findings support our suspicion that individuals may have hereditary hemochromatosis despite relatively low body iron stores as demonstrated by mobilizable iron determinations. Because many of the patients with low body iron stores were identified through screening (prior to the development of end organ damage), we postulated that mobilizable iron may be

correlated with age. In the 37 homozygous C282Y patients who were iron depleted, there was no correlation between age and mobilizable iron (Pearson correlation coefficient 0.15).

Liver biopsies were performed on 25 of these 41 patients; 4 of these patients had a hepatic iron index less than 1.9 while the others were greater. Whereas a hepatic iron index >1.9 is usually seen in hereditary hemochromatosis, 3 of the 4 patients with a low hepatic iron index had a quantitative hepatic iron >50  $\mu\text{mol/g}$  dry weight, representing increased iron stores. One patient had an inadequate biopsy sample. This suggests that the hepatic iron index may not be diagnostic in all cases of hereditary hemochromatosis. The mean

mobilizable iron in this group of patients was 5.15 grams and the mean hepatic iron index was 3.91.

Five patients were compound heterozygotes for C282Y and H63D. Four of these patients underwent liver biopsy; two had a hepatic iron index greater than 1.9 and a third patient with a hepatic iron index of 1.3 had a quantitative hepatic iron of 90.6  $\mu\text{mol/g}$  dry weight. All patients were phlebotomized to a state of iron depletion and only one of these patients had a mobilizable iron greater than 4 grams.

Three of the 61 patients were homozygous H63D. These patients had either an hepatic iron index  $>1.9$  or mobilizable iron stores of greater than 4 grams, thereby meeting standard criteria for a diagnosis of hereditary hemochromatosis. Clinically, they could not be distinguished from the other patients.

Seven patients in the series were heterozygous for either the C282Y or H63D mutation. Of these patients, three had significant iron overload as evidenced by mobilizable iron or hepatic iron index values, while four did not.

Five patients in this series had neither HFE mutation and were categorized as having a normal genotype. One unequivocally has iron overload with a hepatic iron index of 4.4. Another patient had been treated prior to arrival at our center and therefore was already iron depleted. The last 3 patients may have other reasons for a clinical diagnosis of hereditary hemochromatosis. The presence of significant iron loading in some patients with a normal genotype and some heterozygous patients supports the fact that hemochromatosis may occur in the absence of the characteristic HFE mutations.

## DISCUSSION

Throughout the last decade, physician awareness of hereditary hemochromatosis has increased significantly in part due to studies demonstrating that it is a common disorder as well as studies showing that it is cost-effective to

screen for the disorder (5,6). As awareness of hereditary hemochromatosis has increased, a number of questions have emerged regarding the optimal screening strategy, the need for liver biopsy, and most recently, the relationship between HFE genotype and clinical measures of iron stores. The purpose of this study was to analyze the relationship between HFE genotypes and hepatic iron index values and mobilizable iron determinations in a cohort of patients being treated for hereditary hemochromatosis in a uniform fashion at one treatment center.

The initial study describing HFE mutations suggested that 87% of patients with hereditary hemochromatosis have mutations within this gene, with 83% being homozygous for C282Y (16). Most of our patients who were homozygous C282Y had a hepatic iron index  $>1.9$  although a significant number had relatively low mobilizable iron stores. This likely reflects the fact that many of our patients were diagnosed through screening, not by overt clinical evidence of disease. Although we initially thought this was in part a function of age, there was no correlation between mobilizable iron and age. This variability in mobilizable iron may also be a manifestation of variable phenotypic penetrance. Large studies and pooled data from many centers will be required to further clarify penetrance.

Our series concurs with earlier reports showing that the majority of hereditary hemochromatosis patients are homozygous C282Y, but also raises questions regarding patients who are compound heterozygotes (C282Y/H63D) or homozygous H63D and appear to have increased iron stores. These two patient groups are overrepresented in our population (13%), and may reflect our threshold for making a clinical diagnosis of hereditary hemochromatosis in patients with relatively low total body iron burdens. While there is considerable evidence that homozygous C282Y is the predominant genetic mutation leading to hereditary hemochromatosis, the importance of the H63D mutation remains to be clearly defined (18). It has been estimated that the penetrance of

the compound heterozygous state and homozygous H63D is low (19,20). Both published series (21), and unpublished observations (22) suggest that there will be variability in genotypic patterns based on how patients are defined and their country of origin. We await larger studies before drawing conclusions about the overall significance of these genotypes.

Lastly, we have five patients with presumed hereditary hemochromatosis who do not have either HFE mutation. One of these patients has significant iron overload supporting the contention that HFE mutations do not account for all cases of hereditary hemochromatosis. Overall our clinical experience is in keeping with that which is observed in other genetic disorders; phenotypic expression is variable and some patients with the disease may not have the characteristic genotype.

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